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Distribution of Herpes Simplex Virus Types 1 and 2 Genomes in Human Spinal Ganglia Studied by PCR and In Situ Hybridization

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Clinical data indicate that the recurring herpes simplex virus (HSV) from oro-labial lesions is HSV subtype 1 and that the virus from genital lesions is HSV-2. This suggests that HSV-1 and HSV-2 reside in latent forms in the trigeminal ganglia and sacral ganglia, respectively. However, the distribution of latent HSV-1 and HSV-2 infections in human spinal ganglia has not been fully examined. This report concerns the application of polymerase chain reaction (PCR) and in situ hybridization (ISH) to such a study. By using PCR and employing the respective primers, HSV-1 and HSV-2 DNAs were detected in 207 of 524 samples from 262 spinal ganglia (from the cervical to the sacral ganglia) examined on both sides. The percentages of HSV-1 and HSV-2 detected in a given set of ganglia were similar, indicating an absence of site preference. By ISH, few but positive hybridization signals were detected evenly in sacral ganglia sections. The data suggest that regional specificity of recurrent HSV infections is not due to regional distribution of latent virus, but that local host factors may be important for recurrences. ***J. Med. Virol. 52:136–142, 1997.***

KEY WORDS: virus latency; in situ hibridization; HSV-1; HSV-2

INTRODUCTION

Herpes simplex virus (HSV) is classified into two subtypes, HSV-1 and HSV-2. A characteristic feature of HSV is latent infection of ganglion cells. After the first infection, infectious virus particles are not detected, and the virus genome is incorporated into the nucleus of ganglion cells. The incorporation of the viral genome is at the rate of 1–2 copies per 100 ganglion cells [Roizman and Sears, 1996]. Reactivation of a latent HSV infection can occur spontaneously, or in association with physical or emotional stress, fever, exposure to ultraviolet (UV) light, tissue damage, and immune suppression [Whitley, 1996]. However, the mechanism involved in the establishment and maintenance of neuronal latency and the subsequent reactivation of such a latent infection remains uncertain.

Studies of herpes virus skin diseases revealed that HSV-1 was isolated principally from oro-labial lesions innervated by the trigeminal ganglia and that HSV-2 was frequently isolated from lesions in the genital areas innervated by sacral ganglia. Based on these findings it has been assumed that latent HSV-1 and HSV-2 infections persist in the trigeminal ganglia and sacral ganglia, respectively, and that these separate locations seem to be kept strictly [Nahmias et al., 1981]. However, in addition to these 2 major sites, HSV seems to be latent in other sites of spinal ganglia, as herpetic vesicles are seen in the upper extremities, the dorsum of hands, and the gluteal region [Whitley, 1996].

In the earlier studies, evidence of viral latency was obtained by the recovery of the virus in neuronal cultures of human ganglia [Baringer and Swoveland, 1973; Baringer, 1974], with the recent application of in situ hybridization (ISH) verifying the strictly separate viral residence in human ganglia [Lafferty et al., 1987; Stevens et al., 1987; Croen et al., 1987]. Thus it was shown by ISH that the expression of latency-associated transcript of the HSV-2 gene was predominant in the sacral ganglia and that of the HSV-1 gene in the trigeminal ganglia [Croen et al., 1991]. Similar results have been reported by using the polymerase chain reaction (PCR) [Mahalingam et al., 1992]. However, to our knowledge, there has been no systematic examina-

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TABLE I. Cases Studied

Case no.	Age/Sex	Pathologic diagnosis	Hours post-mortem	HSV antibody test	PCR and/or ISH
case 1	77/F	PSS	1:21	+	PCR
case 2	56/M	TCC	1:23	+	PCR
case 3	65/F	NHL	3:12	+	PCR
case 4	72/M	Colon cancer	2:10	+	PCR
case 5	47/M	Small cell lung cancer	1:20	ND	PCR
case 6	63/F	Autoimmune-disease	2:40	ND	PCR
case 7	39/M	CML	1:30	+	PCR
case 8	78/F	Aplastic anemia	3:20	ND	PCR and ISH
case 9	53/M	Malignant lymphoma	2:30	ND	PCR and ISH
case 10	70/M	Gastric cancer	1:33	ND	PCR
case 11	66/F	Thyroid cancer	3:05	ND	ISH

F: female. M: male. PSS: Progressive systemic sclerosis. TCC: Transitional cell carcinoma of the urinary system. NHL: Non-Hodgkin's lymphoma. CML: Chronic myelocytic leukemia. ND: not done.

tion of the human spinal ganglia (from the cervical ganglia to the sacral ganglia) to detect HSV-1 and HSV-2 by the use of PCR and the respective specific primers. We describe the results of applying PCR and ISH to the study of 294 spinal ganglia from 11 autopsied patients. Our data indicate that latent infections of both HSV-1 and HSV-2 are present throughout the spinal ganglia in an apparently even fashion, with no type-specific viral residency.

MATERIALS AND METHODS

Spinal ganglia were taken initially from 7 individuals during consecutive autopsies performed at Hokkaido University Hospital. Not all ganglia were obtained from a given autopsy, and some could not be taken due to difficulties with macroscopic recognition. The ganglion samples were divided into 2 groups. One group, to be used for PCR, was frozen in liquid nitrogen and stored at −70°C prior to DNA extraction, and the other, to be used for ISH, was immersed in 10% buffered formalin or 4% paraformaldehyde, paraffin embedded and stored at 4°C. There were difficulties in obtaining the distal sacral ganglia from usual autopsies, particularly the fourth and fifth sacral ganglia which innervated the genital regions. To overcome this problem, we dissected the sacral bones, together with spinal nerves and sacral ganglia on block from 4 individuals, and carefully exposed the sacral ganglia by destroying the bone piece by piece. Although the presence of thin fifth sacral nerves was recognized, the fifth sacral ganglion was not evident in any case. Two sacral ganglia obtained (cases 8 and 9) were used for PCR and ISH, one (case 10) for PCR only, and the other (Case 11) for ISH only. Table 1 summarizes the 11 cases investigated. The average age at death was 62.3 years; 6 were males, 5 were females. Sera taken at autopsy from the right ventricle were separated from formed elements by centrifugation at 3,000 rpm, stored at −20°C, and used for virus antibody tests. An antibody was examined by neutralizing and an IgG enzyme immunoassay at the Sapporo Clinical Laboratory Center (Sapporo, Japan).

Polymerase Chain Reaction

High molecular weight DNA was extracted from each ganglion by the phenol-chloroform method [Maniatis et al., 1989]; 1 μg samples were used. DNA was amplified by PCR using the HSV-1 and HSV-2 specific primer pairs [Saiki et al., 1988]. The presence of amplified HSV-1 and HSV-2 DNAs was determined as previously described [Kimura et al., 1990]; type-specificity was dependent on the downstream primers used. The HSV-1 primers were DNAP5 (5′-ATGGTGAACATCGACATG-TACGG-3′) and DNAP3-1 (5′-CCTCGCGTTC-GTCCTCGTCCTCC-3′) and the HSV-2 primer pair was DNAP5 and DNAP3-2 (5′-CCTCCTTGTCGAGGC-CCCGAAAC-3′). With these primers, a 469 bp product is obtained for HSV-1 and a 391 bp product for HSV-2. As the internal control, human α-tubulin gene [Cowan et al., 1983] was also amplified by PCR, using these primers: TUB1 (5′-GACAGAATTCCAGACCAACC-3′) and TUB2 (5′-GCACCAATCCACAAACGTGA-3′), which yield a 286 bp product. Detection of amplified α-tubulin DNA was performed with a method described previously [Takasu et al. 1992]. For each sample, 1 μg of tissue DNA was amplified on a thermal cycler (Astec, Japan) with Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) in a total volume of 100 μl. All other conditions were as described previously [Kimura et al., 1990; Takasu et al., 1992].

Southern Blot Hybridization

Each 10 μl PCR product was separated by electrophoresis on a 2% agarose gel or agarose gel of 1% Seakem/3% NuSive (FMC BioProducts, Rockland, ME), and blotting was carried out as described previously [Suzuki et al., 1993]. As positive controls we used PCR-amplified DNAs from HSV-1 strains K192 and K193, and HSV-2 strains 20 and 27 (kindly provided by Dr. R. Hondo of the Institute of Public Health, Tokyo). High molecular non-amplified DNA (1 μg) of case 2 was used as a negative control. Filter was processed for hybridization with ^{32}P-labeled or digoxigenin-labeled HSV DNA probes.

The ^{32}P-labeled probe obtained from DNA amplified

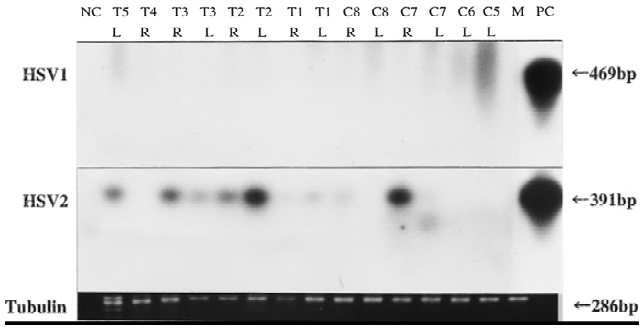

Fig. 1. Analysis of portions of the spinal ganglia of Case 3 for HSV-1 and HSV-2 DNAs. **Top:** Cervical (C) and thoracic (T) ganglia DNAs were subjected to PCR amplification with the HSV-1 primer pair, and the products analyzed by Southern blotting. No 469 bp product of HSV-1 DNA was detected. **Middle:** DNAs from the same ganglia were amplified with the HSV-2 primers and analyzed by Southern blotting. The 391 bp product of HSV-2 DNA was detected in C7L, C7R, C8R, T1L, T1R, T2L, T2R, T3L, T3R, and T5L. **Bottom:** The PCR product obtained with the α-tubulin primers was electrophoresed on a 2% agarose gel, and stained with ethidium bromide. PC, positive control; NC, negative control; L, left. R, right; M, size marker (ϕX174/*Hae* III digest).

with HSV-1 strain K193 DNA (megaprime kit, Amersham, UK) detects both HSV-1 and HSV-2 signals as this probe includes sequences common to HSV-1 and HSV-2. Hybridization was carried out as described previously [Suzuki et al., 1993], and autoradiography was carried out with Kodak X-Omat film.

The probe labeled with digoxigenin-11-dUTP was obtained from DNA prepared by additional 10 PCR cycles using as template 100 ng DNA of PCR-amplified HSV-1 or HSV-2 DNA (DNA labeling and detection kit, Boehringer Mannheim, Germany). Hybridization was carried out as described previously [Takasu et al., 1992]. The signals were detected using Boehringer Mannheim labeling and detection kit following manufacture's protocol.

In Situ Hybridization

HSV-1 DNA was obtained consisting of a 2.6 kb *Sal*I/*Bam*HI DNA fragment derived from the *Bam*HI-B restriction fragment of HSV-1 [Furuta et al., 1992] and HSV-2 DNA consisting of a 3.4 kb *Bam*HI-P restriction fragment of HSV-2 [Croen et al., 1991]. These DNA fragments contain the infected-cell protein number zero (ICPO) gene and the latency-associated transcript (LAT) gene (see Fig. 2 of Furuta et al., 1992, and Fig. 1 of Croen et al., 1991). The HSV-1 2.6 kb *Sal*I/*Bam*HI DNA fragment was cloned into pSPT18, which contains SP6 and T7 RNA polymerase promoters. The HSV-2 3.4 kb *Bam*HI-P restriction DNA fragment was cloned into pBluescript sk(+), which contains T3 and T7 RNA polymerase promoters. The RNA probes were synthesized by in vitro transcription in opposite directions to that of ICPO mRNA (LAT sense probe) and LAT (LAT antisense probe) [Maniatis et al., 1989]. The RNA probes were labeled with digoxigenin-UTP and then subjected to limited alkaline hydrolysis to reduce their size to an average of 200 nucleotides.

Thin sections (5 μm) of paraffin-embedded tissues were mounted onto slides coated with 3-aminopropyltriethoxysilane. The tissues were deparaffinized and rehydrated by sequential immersion in xylene and decreasing concentrations of ethanol. The sections were then treated with 0.2 N NaCl for 20 min and 100 μg/ml proteinase K (Boehringer Mannheim) at 37°C for 15 min. After post-fixation with 4% paraformaldehyde in PBS for 5 min, excess aldehyde was neutralized by two 15-min treatments with 0.2% glycine in PBS, followed by washing of the sections in PBS. The slides were acetylated with 0.25% acetic anhydride (in 0.1 M triethanolamine) for 10 min, the sections dehydrated by sequential immersion in ascending concentrations of ethanol (50%, 70%, 90%, and 100% twice) and the slides dried in air. Hybridization was performed at 50°C for 15 h in a solution consisting of 50% deionized formamide, 600 mM NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, 10 mM dithiothreitol, 1 × Denhardt's solution, 0.25% SDS, 200 mg/ml *Escherichia coli* tRNA, and 200–1000 ng/ml of digoxigenin-labeled probe. The slides were washed in 0.1 × SSC at 60°C, immersed in 0.5% blocking reagent (Boehringer Mannheim) for 30 min, and then washed in DIG buffer 1. Subsequently the slides were incubated for 30 min with alkaline phosphatase-conjugated anti-digoxigenin antibody and diluted 1:5000 in DIG buffer 1. They were then washed twice with DIG buffer 1 for 15 min, washed twice with Buffer 2 (100 mM Tris-HCl, 100 mM, 10 mM NaCl, pH 7.5) for 10 min, and incubated for 30 min in HNPP/Fast red TR (3-hydroxy-N-2′-biphenyl-2-naphthalenecarboxamide phosphate ester/4-chloro-2-methylbenzene-diazonium salt) solution (Boehringer Mannheim) [Kagiyama et al., 1995; Kondoh et al., 1995]. The slides were then dipped in buffer 2 and covered with coverslips. Fluorescent signals were detected with a Nikon fluorescence microscope equipped with a D-F-T filter set (Tokyo, Japan). As positive controls of HSV-1 we used a human trigeminal ganglion that had been found to be positive by ISH using radioisotope and digoxigenin [Furuta et al., 1992; Takasu et al., 1993], and for HSV-2, mice brain infected with the Ogiwara strain of HSV-2 [Nii and Yasuda, 1981].

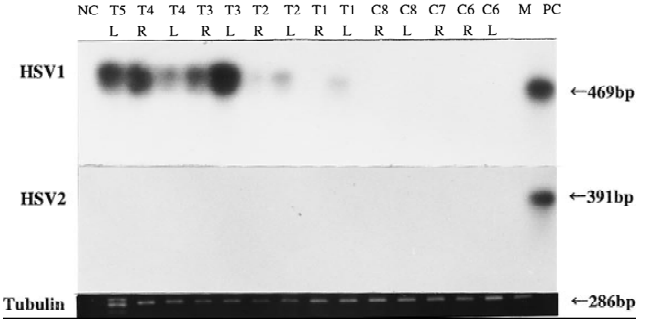

Fig. 2. Analysis of cervical and thoracic spinal ganglia of case 4 for HSV-1 and HSV-2 DNAs. A 469 bp product of HSV-1 DNA was detected in T1L, T2L, T2R, T3L, T3R, T4L, T4R, and T5L (**top**). No 391 bp product of HSV-2 DNA was found (**middle**). For abbreviations, see Figure 1.

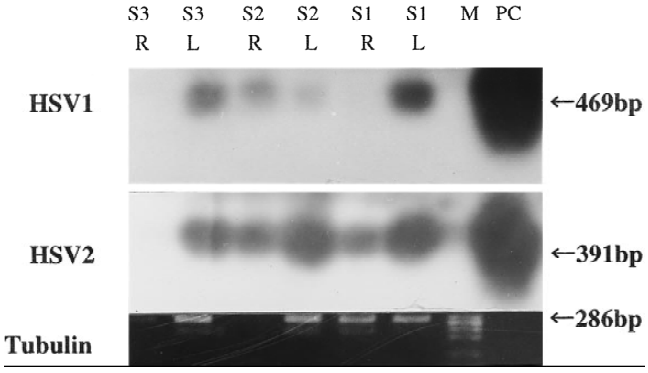

Fig. 3. Analysis of sacral ganglia of case 9 for HSV-1 and HSV-2 DNAs. HSV-1 DNA was detected in S1L, S2L, S2R, and S3L, and HSV-2 DNA in S1L, S1R, S2L, S2R, S3L, and S3R. For abbreviations, see Figure 1.

RESULTS

PCR and Detection of Amplified HSV-1 and HSV-2 DNAs by Southern Blot Hybridization

DNA bands of both HSV-1 (469 bp) and HSV-2 (391 bp) were detected with the respective positive controls; no band was seen with the negative controls (Figs. 1 and 2). These findings indicated that specific PCR-mediated gene amplification had taken place and that the PCR-Southern blotting method could estimate latent HSV infection. Of a total of 294 ganglia, 32 were excluded from further analysis due to absence of α-tubulin DNA amplification, used as the internal positive control. Of the remaining 262 ganglia, 207 (39.5%) were positive for either HSV-1 or HSV-2; HSV-1 was detected in 104 and HSV-2 in 103. Despite the variations from case to case, the overall distribution of HSV-1 and HSV-2 in the specimens of the 10 individuals showed no differences in regard to ganglia. HSV-1 and HSV-2 were detected in 11/40 (27.5%) and 15/40 (37.5%) cervical ganglia; 52/127 (40.9%) and 46/127 (36.2%) thoracic ganglia; 16/53 (30.2%) and 16/53 (30.2%) lumbar ganglia; and 25/42 (59.5%) and 24/42 (57.1%) sacral ganglia, respectively. In addition, in the 3 cases of the sacral ganglia (cases 8–10), both HSV-1 and HSV-2 were detected in a high percentage of cases (Fig. 3); HSV-1 was found in 22/23 ganglia, and HSV-2 in 21/23. Our overall data indicate that latent HSV-1 and HSV-2 infections are widespread and distributed evenly from the cervical to the sacral ganglia. No laterality in HSV distribution was evident (Table II).

There were, however, some differences in distribution among the cases studied. For example, in case 1, both HSV-1 (6/14 from the left ganglion and 11/20 from the right; total = 17/34, 50.0%) and HSV-2 (5/14 from the left and 8/15 from the right; total = 13/29, 44.8%) were distributed evenly in both sides from the cervical ganglia to the sacral ganglia. Similar distribution patterns were seen with cases 2, 5, 6, and 7. In contrast, HSV-2 was the dominant persisting type in case 3, with HSV-1 found in 1/33 ganglia (3%) and HSV-2 in 20/34 (58.8%) (Fig. 1). Alternatively, HSV-1 predominated in case 4; it was detected in 10/34 (29.4%) ganglia, while HSV-2 was not identified in any of them; most HSV-1 was detected in the upper thoracic ganglia (Fig. 2).

TABLE II. Results of Polymerase Chain Reaction

	Case 1		Case 2		Case 3		Case 4		Case 5		Case 6		Case 7		Case 8		Case 9		Case 10	
HSV	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
	LR	LR	LR	LR	LR	LR	LR	LR	LR	LR	LR	LR	LR	LR	LR	LR	LR	LR	LR	LR
C4											−−	−−								
C5					−	−			−	−	−−	+−	++	−+						
C6	+−	++	++	++	−	−	−−	−−	−	+	−−	−−	−	−						
C7	−	+	+	+	−−	++	−	−	−−	−−	−−	−−	+	−						
C8	+	+	+−	++	−−	−+	−−	−−	−	−	−	−	++	−−						
Th1	−−	+−	−−	−−	−−	++	+−	−−	−	−	−−	−−	+	+						
Th2	+−	−+	++	++	−−	++	++	−−			−+	+−								
Th3			++	++	−−	++	++	−−	−	−	+	−								
Th4	−−	+	++	++	−	−	++	−−	+	−	++	−−	+−	−−						
Th5			−−	−−	−	+	++	−−	−	+	++	+−	+	+						
Th6			−−	−+	−	+					++	−−	+	+						
Th7	+	−	+−	+−	−−	++	+−	−−	−−	+−	−−	−−	−−	−−						
Th8	+	+	−	+	−−	−−	−−	−−	++	+−	−−	−−	−−	−−						
Th9	++	+−	−−	++	+	+	−−	−−	−	−	−+	−−	−−	+−						
Th10	++	+−	++	++					++	−−	−	−	−−	−−						
Th11	+−	−−	++	++	−−	+−	−−	−−	−−	−−	−−	+−	++	−−						
Th12	−−	+−	+	+	−	+	−−	−−	−−	−−	−−	−−	+	−						
L1	−+	−+	−+	−+	−	+	−−	−−	−−	−−	−−	+	++	−+						
L2	−−	−−	−+	++	−−	++	−	−					+−	++						
L3	−−	−−	+−	−−	−−	+−	−−	−−	−+	+−	−	−	++	+−						
L4	−−	−+			−	−	−−	−−	+	+	−−	−−	++	−−						
L5			+	+	−−	−−	−−	−−	−	−	+−	−−	+	−						
S1	−	−	−−	−−	−	−					−−	−−	−−	−−	++	++	++	+−	++	++
S2	−	−	−+	−−	−−	−−					−−	++	+	−	++	++	++	++	++	++
S3											−	+	+−	−−	+−	++	++	++	++	++
S4															+	+	++	++	++	−+

L, left; R, right; C, cervical; Th, thoracic; L, lumbar; S, sacral.

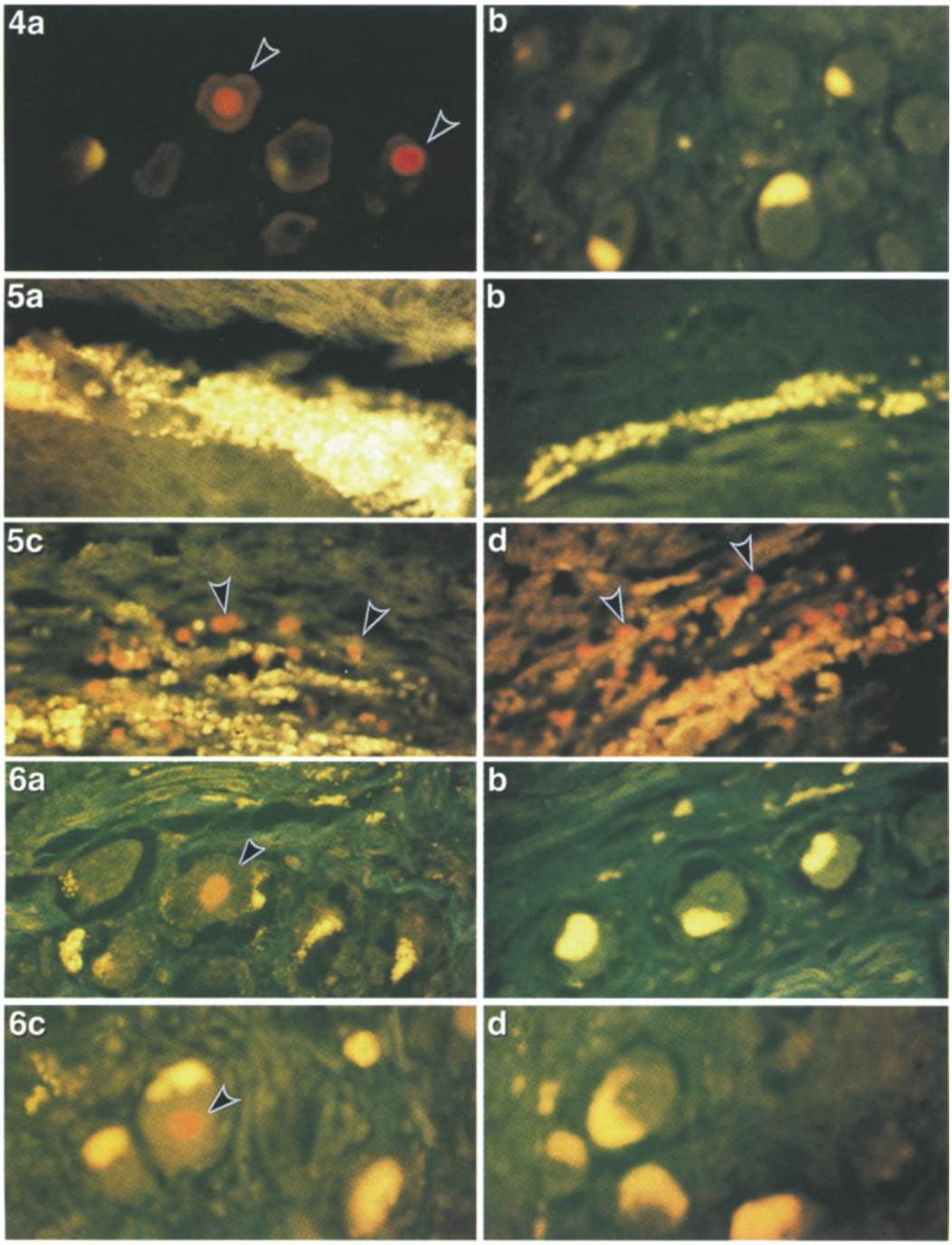

Fig. 4. In situ hybridization for HSV-1 LAT in the trigeminal ganglia, found to be positive in a previous study [Furuta et al., 1992]. Positive red fluorescence hybridization signals are observed in the nuclei of neurons with the HSV-1 antisense probe (**4a**); no hybridization is seen with the sense probe (**4b**). The yellow color is autofluorescence of lipofuscin pigments. × 200.

Fig. 5. HSV-2 infected mouse brain used for in situ hybridization tests. Both antisense (**5a**) and sense (**5b**) HSV-1 probes failed to hybridize, while the antisense (**5c**) and sense (**5d**) HSV-2 probes gave positive signals. These control experiments showed that the probes used in our study were HSV type specific. × 200.

Fig. 6. In situ hybridization of HSV-1 and HSV-2 in the fourth sacral ganglia of case 9. Positive hybridization signals with the HSV-1 antisense probe are seen in a few neuronal nuclei (**6a**), while none is evident with the HSV-1 sense probe (**6b**). A few sacral neurons are also hybridized with the HSV-2 antisense probe (**6c**), but not with the HSV-2 sense probe (**6d**). × 200.

Antibodies Against HSV-1 and HSV-2

The presence of antibodies against HSV-1 and HSV-2 in the sera taken at autopsy was determined by a neutralization test and to HSV-1 by IgG enzyme immunoassay. Our results indicated that all cases examined had antibodies to both HSV types, although these tests may not always be able to differentiate strictly the type difference (data not shown).

In Situ Hybridization

A red fluorescence signal was detected in the nuclei of large sensory neurons of the trigeminal ganglia used as positive HSV-1 controls after hybridization with the HSV-1 antisense RNA probe, but not with the sense probe (Fig. 4). However, with mouse brain infected with HSV-2, positive hybridization signals were observed with both the antisense and the sense RNA probes for HSV-2, but not with those for HSV-1 (Fig. 5). Because during acute productive HSV-2 infection sense and antisense RNAs are transcribed [Croen et al., 1987], our findings with the two HSV-2 probes on infected mouse brain were not unexpected. Consequently, the results of the control studies indicated that the probes employed in our investigation were type-specific for HSV-1 and HSV-2. When the HSV-2 antisense probe was applied to an HSV-1-positive trigeminal ganglion, a few nuclei had a positive signal (data not shown), suggesting that HSV-2 latently resides in trigeminal ganglia. A few definite hybridization signals with both the antisense HSV-1 and HSV-2 probes were detected in the sacral ganglia (Fig. 6); hybridization signals were not detected with the sense probes. Moreover, positive signals were not seen in satellite cells or in other non-neuronal cells of the ganglia. It was found that the LAT of HSV-1 was present not only in trigeminal ganglia, but also in sacral ganglia, and that the LAT of HSV-2 was evident in sacral ganglia as well as in trigeminal ganglia. Thus the ISH findings confirmed that the presence of the respective DNAs detected by PCR represent latent infection by HSV-1 and HSV-2.

DISCUSSION

Latent infections of human trigeminal and spinal ganglia by HSV-1 and HSV-2 were verified recently using molecular biology techniques. Mahalingam et al. (1992) applied PCR to examine the thoracic and trigeminal ganglia of 15 autopsy cases finding that HSV-1, but no HSV-2, was present as a latent infection. Croen et al. [1991] in studying 10 sacral ganglia from 5 individuals by ISH only found positive signals when probing for HSV-2. However, they also found that 3 of 50 sacral ganglia gave hybridization signals for HSV-1, stating that this could represent cross-hybridization due to HSV-1 and HSV-2 gene homology. From earlier co-cultivation studies, it had been accepted [Baringer and Swoveland, 1973; Baringer, 1974] that HSV-1 lodges in the trigeminal ganglia and HSV-2 in the sacral ganglia. However, our data show that latent HSV-1 and HSV-2 infections are widespread and distributed evenly from the cervical ganglia to the sacral ganglia without a virus type exhibiting a site of predilection.

Because PCR is an extremely sensitive technique that can amplify even contaminating DNA templates, ISH was used to document directly the presence of HSV latency-associated transcripts. Positive hybridization signals were readily detected in the nuclei of the trigeminal ganglia and the sacral ganglia, thereby confirming the PCR results and verifying that HSV-1 and HSV-2 latency was distributed among the spinal ganglia with no type-specific residency. The demonstration of HSV-2 in human trigeminal ganglia is compatible with the experimental data on the latent infection of these ganglia by HSV-2 [Kurata et al., 1978].

As with most recurrent viruses of oro-labial herpes are HSV-1 and those of genital herpes are HSV-2 [Roizman and Sears, 1996; Whitley, 1996], it is assumed that the localization specificity of recurrent HSV infections is due to local host factors that may be implicated in virus reactivation. It can be argued that dissimilarities in the nucleotide sequences of HSV-1 and HSV-2 could predict the appropriate differences in virus preference for cells lining the oral and genital mucous membranes. Answers to this require isolation of local transcriptional factors that would activate specifically each viral promoter. It was found that trigeminal ganglia used as positive controls for HSV-1 had a few trigeminal neurons that gave HSV-2 positive hybridization signals. Because it was thought that almost all trigeminal neurons were latently infected with HSV-1, it is important to determine the rate of HSV-2 latency in trigeminal ganglia by analyzing many samples. In addition, it would also be of considerable interest to ascertain whether both virus types lodge in these ganglia, and if a single neuron can be the simultaneous host for HSV-1 and HSV-2.

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